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Immuno-monitoring reveals an extended subclinical disease activity in tocilizumab-treated giant cell arteritis

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Abstract

Objective: Tocilizumab is effective in inducing and maintaining remission of Giant Cell Arteritis. Despite clinical and serological control of disease, magnetic resonance angiography may show persistence of inflammatory signals in arterial walls of unknown significance. Thus, there is an unmet need for tools to detect subclinical disease activity.

Methods: Immune-inflammatory markers in prospectively collected sera of the first RCT about tocilizumab in Giant Cell Arteritis were measured. As comparison sera of age- and sex-matched healthy volunteers were used. Biomarkers were quantified using luminex technology.

Results: From all parameters determined only MMP-3, pentraxin-3 and sTNFR2 were significantly elevated while ICAM-1 and CD163 were significantly decreased at early stages of the study, at time points of full clinical remission under treatment with tocilizumab plus glucocorticoids. In contrast, tocilizumab monotherapy towards the end of the study resulted in an almost complete normalization of immune-inflammatory molecules as compared to healthy controls. MMP-3 levels showed a weak association with magnetic resonance signal intensity; none of the biomarkers predicted relapse occurring within 6 months after study end.

Conclusion: The data document a subclinical disease activity in GCA which is more pronounced at early stages of treatment and almost disappears towards study end. They indicate that TCZ treatment of at least 52 weeks is necessary to reset a broad range of immune-inflammatory pathways.

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Key words: giant cell arteritis, tocilizumab, glucocorticoids, biomarkers, disease activity, MMP-3

Key messages:

- Serological biomarkers document an extended subclinical disease activity in treated GCA during early remission
- Prolonged inhibition of the IL-6 pathway using TCZ is not only abolishing signs and symptoms of the systemic inflammation but appears to reset a broad range of inflammatory mechanisms in GCA

Clinical trials registration: ClinicalTrials.gov NCT01450137

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Introduction

Giant Cell Arteritis (GCA) is the most frequent large vessel vasculitis (LVV) in Western countries, affecting predominantly women over 50 years of age [1,2]. It is histologically characterized by intense inflammation in all layers of the medium- to large-sized arteries and formation of granulomas. Risks are obliteration of the vascular lumen resulting in blindness and aneurysm formation with rupture of the aorta. Conventional treatment consists of high-dose and long-term glucocorticoids (GC), leading to side effects over time. Methotrexate helps to reduce the risk of relapse and to spare GCs [3]. Tocilizumab (TCZ) has very recently been proved to maintain remission and to lead to highly significant reduction of the cumulative GC dose [4,5].

Between 2012 and 2015 we performed the first phase 2, randomised, double blind, placebo-controlled trial (RCT) to define the role of TCZ to induce and control remission of GCA (ClinicalTrials.gov registration number: NCT01450137) [4]. Whereas all but one patient in the TCZ arm stayed in lasting remission up to study end at week 52, only 2 patients in the placebo arm did not relapse.

Patients with initial positive MR-angiography (MRA findings) underwent control MRA at weeks 12 and 52. At week 12, three out of 9 TCZ patients (33%) showed normalization of vessel wall signals. At week 52, there was additional MRA improvement in some TCZ patients, but one-third showed persistent or increased late vessel wall enhancement despite clinical remission [6]. The significance of these “inflammatory” signals is currently unknown.

The protocol of the RCT included sampling of sera at the beginning and prior to every 4-weekly infusion, offering a unique opportunity to study a broad range of immune-inflammatory biomarkers prospectively in a very well characterized patient population. This was of particular interest as remission was initially induced by a combination of GC plus TCZ and maintained towards study end by TCZ in monotherapy.

The aims of this study were i) to search for and characterize a potential subclinical disease activity ii) to compare the biomarker profiles in early full remission under combined therapy with TCZ plus GCs and at late stage under TCZ monotherapy, iii) to search for markers associated with persistent MRA signals in vessel walls and iv) for markers predicting relapse within 6 months after study end.

Methods

Patients and Controls

Thirty patients aged 50 years or older suffering new-onset or relapsing GCA, confirmed by either temporal artery biopsy or by MR angiography (MRA), and meeting the 1990 American

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College of Rheumatology criteria were included in the RCT [4]. Of these, 20 received TCZ which was dosed as to treat rheumatoid arthritis, i.e. 8mg/kg bodyweight i.v. in 4-weekly intervals. GC co-medication was started at a dose of 1mg/kg bodyweight and rapidly reduced thereafter: -0.1mg/kg bodyweight weekly until week 8, then -0.05mg/kg bodyweight weekly until week 12, followed by monthly -1mg/day. This reduction scheme resulted in a mean daily dose of 7mg prednisone at week 12 and a discontinuation of GC between months 9 and 11. A prior treatment with prednisolone up to 1mg/kg bodyweight for a maximum of 10 days between inclusion in the trial and the first infusion was permitted. Blood samples were collected at weeks 0, 2, 4, 6, 8, 10, 12 and in 4-weekly intervals thereafter until week 52. The serum samples were immediately processed and stored in aliquots of 0.5ml at -70°C.

Sera of TCZ patients with relapse (N=1), serious adverse events (N=2) or with incomplete sampling (N=3) were excluded from analysis. Therefore, the longitudinally analyzed cohort comprised 14 TCZ patients in lasting remission for 52 weeks. Sera of patients in the placebo arm were purposely not analyzed as GC treatment had to be re-increased repeatedly, resulting in a very inhomogeneous patient population.

For every patient a serum of an age- and sex-matched healthy control was collected. These individuals lived on their own, they did neither take any regular medication, anti-inflammatory agent nor did they show signs of infection within 4 weeks of venipuncture.

Biomarkers and Methods

Based on recent studies and pathophysiological considerations a broad range of biomarkers was chosen (suppl. table). This included proteins of the TNF superfamily, of the IFN family, of T lymphocytes and regulatory T cells, monocytes, B lymphocytes, inflammatory markers such as hsCRP, adipocytokines and catabolic enzymes such as metalloproteinases (MMPs). Investigations were performed with luminex technology (R&DSystems, Minneapolis, USA and Invitrogen, Carlsbad, USA). Samples were quantified on a multiplex system (Bio-Plex 100 array reader with Bio-Plex Manager software [version 6.1]; Bio-Rad, Hercules, CA, USA). If not specified, protein concentrations are given in pg/ml. In addition, TCZ, IL-6 and sIL-6 concentrations were determined by an extern company using luminex technology (QPS, Groningen, The Netherlands).

MR-angiography and relapses

MR signals were analyzed as described elsewhere [6]. In brief, MR angiography was repeated at 3 months and at study end if patients showed signs of mural inflammation at recruitment. Vessel wall signals were judged by two experienced radiologists in blinded fashion using the following grading: 0 = no mural thickening (maximal vessel wall thickness < 2.3 mm), no enhancement; 1 = no thickening, slight mural enhancement; 2 = mural thickening (> 2.3 mm),

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significant mural enhancement; 3 = strong thickening (> 3 mm). Correlations were calculated between biomarkers and 21 MRA signals (7 patients treated with TCZ and available biomarkers with MRA examinations at all 3 time points).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software and IBM SPSS Statistics 21. Welch's correction was used to calculate significance between paired sample groups and Mann-Whitney U test between unpaired sample groups. Correlation analysis was done by calculation of Spearman rank's correlation coefficient. To search for biomarkers predicting flare and mural signal intensity, binary logistic regression and univariate receiver operating characteristic (ROC) curves were generated.

Ethical approval and patient informed consent

The amendment to study 168/10 (ClinicalTrials.gov registration number: NCT01450137) was approved by the ethical commission of Bern, Switzerland, the 4. of May 2016 and the study was done in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Results

TCZ, IL-6 and sIL-6R

TCZ levels approached steady state levels by the time of the 6th or 7th dose (figure 1A). Mean pre-dose levels of IL-6 initially increased in response to treatment with TCZ, and subsequently decreased to 50.83 ± 43.08 by week 52 (figure 1B). Levels of sIL-6R levels increased almost immediately after initiation of dosing and appeared to reach a plateau after the 5th or 6th dose (figure 1C).

Biomarkers not detectable in sera or without dynamic changes over time

Eighteen molecules were not present at detectable levels (suppl. table). Remarkably, T-cell cytokines such as IL-2, IL17A and IFN-γ were neither detectable prior to the first TCZ infusion nor at study end.

Several of the measured molecules showed a tendency to fluctuate but did not display a significant increase or decrease over time (suppl. table). Values of sTNFR2 and ICAM-1 did not show dynamic changes but differed compared to healthy controls (figure 2B). Osteopontin levels were slightly higher at study start and they became indistinguishable from controls at study end (P=0.44; not shown).

Biomarkers with significant changes over time

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Pentraxin-3 and MMP-3 serum levels showed a remarkable decrease while CD163 exhibited a continuous increase over time, reaching almost normal values at the end of the study (figure 2A). Pentraxin-3 started at a median concentration of 6342 (95% CI: 3965 - 8720) and decreased to 2807 (95% CI: 1993 - 3621; p=0.0002). MMP-3 decreased from a median concentration of 76370 (95% CI: 65721 - 87018) to 27642 (95% CI: 21207 - 34077; p=0.0001). MMP-3 levels showed a very homogeneous pattern with little inter-individual variation. At week 4 MMP-3 values were indistinguishable from values before first TCZ infusion, thereafter a steady decrease could be noted to values close to normal. On the other hand, CD163 started at a median concentration of 892198 (95% CI: 628448 – 1155947), increased over time and equaled levels of healthy controls at the end of the study with a median concentration of 1342169 (95% CI: 931206 - 1753133; p=0.0012).

Comparison between sera concentrations at study end and matched healthy controls

Despite complete remission of disease, MMP-3, pentraxin-3 and sTNFR2 levels remained elevated, while ICAM-1 concentrations remained below levels of healthy matched controls (figure 2). At the end of the study the median serum concentration of MMP-3 (27642; 95% CI: 21207 – 34077) remained higher compared to controls (19400; 95% CI: 14622 - 24178, p=0.0353). The same applies to pentraxin-3 (2807; 95% CI: 1993 – 3621) compared to controls (1491; 95% CI: 877 – 2105, p=0.0052) and to sTNFR2 (267; 95% CI: 212 - 322) compared to controls (147; 95% CI: 120 – 174, p=0.0023). The median serum concentration of ICAM-1 (1497851; 95% CI: $1.11x10^6$-$1.89x10^6$) on the other hand stayed below the median serum concentration of controls (2246971; $1.83x10^6$ – $2.66x10^6$, p=0.0134).

Correlation between biomarkers and MRA signals

An association was found between MMP-3 concentration and the MRA signal intensity of the aortic wall as determined by MR angiography, but it did just not reach the level of significance (R^2_{adj}=0.13492, p=0.0566; suppl. figure 1). Calculation of the area under the curve (AUC) gave not a significant result (0.821; 95% CI: 0.616 – 1.000, p=0.093). A serum concentration of MMP-3, equal or higher than 48843 was associated with a high mural signal intensity (sensitivity of 77% and specificity of 100%). Values equal or higher showed an effect size of f^2=0.506, reflecting a strong effect of MMP-3 on mural signal intensity according to Cohen. But the chance to have a high mural signal intensity was not significantly higher with serum concentration equal or higher than 48843.

Biomarkers associated with relapse after study end

Patients relapsing within 6 months of study end are marked in red and with arrows in figure 2. Lower serum concentration of IL-6 and higher serum concentrations of pentraxin-3 were associated with a higher risk of a flare after study end. The AUC for IL-6 reached 0.764 (95%

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CI: 0.523-1.000, p=0.068). A serum concentration of IL-6, equal or higher than 29.45 was slightly associated with flare after study end (sensitivity of 87.5% and specificity of 55.6%). The relative risk to flare after study end was 4.44 times higher for serum concentration over 29.45. For pentraxin-3 the AUC reached 0.646 (95% CI: 0.372-0.920, p=0.312). A threshold of 2241 showed a sensitivity of 66.7% and a specificity of 62.5%. The relative risk to flare after study end was 1.88 times for a serum concentration over 2241.

Correlation of different biomarker and TCZ serum levels

Table 1 shows correlations between six biomarkers and TCZ: IL-6 concentrations directly correlate with TCZ levels (r=-0.5585, p<0.0001), while MMP-3 and pentraxin-3 correlates inversely with TCZ serum level (MMP-3: r=-0.5806, p<0.0001, and pentraxin-3: r=-0.3855, p=0.0044, respectively).

Several direct and inverse correlations between the six biomarkers could be observed: MMP-3 and pentraxin-3 showed an inverse correlation with IL-6 (r=-0.6119, p<0.0001 and r=-0.4158, p=0.0033, respectively). MMP-3 and pentraxin-3 serum levels were directly correlated to each other (r=0.4637, p=0.0005). sTNFR2 also correlated inversely with IL-6 (r= -0.3748, p=0.0087), while CD163 demonstrated a positive correlation with IL-6 (r=0.3327, p= 0.0208). In agreement with the positive correlation between CD163 and IL-6, pentraxin-3 and CD163 showed an inverse correlation to each other (r=-0.0.2826, p=0.0403). ICAM-1 was the only biomarker which showed neither a correlation to TCZ serum level nor to other biomarkers.

Discussion

Two recent RCTs showed effectiveness of TCZ in controlling GCA [4,5]. Both documented a highly significant and clinically relevant sparing effect on GCs. Despite these convincing data, questions beyond the clinical outcome remain. In particular MRA documented persistence of late-enhancement in arterial wall of several patients [6]. It is currently unknown, whether these signals reflect arterial wall inflammation or rather represent persistent hyperaemia. The quantification of a wide range of immune inflammatory markers may shed light on potential subclinical disease, explain MRA signals and predict relapse after study end.

Due to the fact that all but two patients in the placebo arm suffered relapse(s), the GC doses had to be re-increased repeatedly resulting in a highly heterogeneous population. As a consequence the placebo arm could not serve as a meaningful control. Thus, the presented data characterize the longitudinal evolution of subclinical biological activity only. Whether GC monotherapy would have resulted in comparable changes is not known.

The rise in IL-6 levels after the first dose is typically seen after administration of TCZ and is hypothesized to occur due to displacement of IL-6 as TCZ binds to the soluble and membrane bound IL-6 receptors [7]. The slow increase of TCZ trough levels implicates a delayed control

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of the disease by the biologic agent and suggests a central therapeutic role of GC at early stages of the study. Remarkably, the only relapse in the TCZ arm happened in week 11 at a time when GC were substantially reduced and TCZ had not yet reached steady state trough levels. New treatment protocols should respect these findings.

Soluble TNFR2 was proposed as marker of subclinical disease activity in a variety of autoimmune diseases [8,9,10]. In GCA, however, its performance has not been analysed. In this study sTNFR2 levels fluctuated over time and they did not predict relapse. The inter-individual differences are in line with the literature, however, the intra-individual changes remain unexplained. Pentraxin-3 levels have been shown to be associated with vascular inflammation in GCA identifying patients with very recent optic nerve ischemia or recent diagnosis [11]. The values of our study show a steady decrease over time with near normalisation at study end. ICAM-1 concentrations, on the other hand, remained below levels of healthy controls throughout the study. An earlier study documented a rapid fall of ICAM-1 concentration upon GC treatment which persisted over the whole study period and reflected clinical remission [12]. The low ICAM-1 levels at start of our study may be explained by the fact that patients were allowed for GC treatment before first TCZ infusion of up to 10 days. Findings of a more recent trial about infliximab to treat GCA showed increased levels of ICAM-1 near relapse [13]. As our patients did not show relapses, our findings are in good agreement with these data and they suggest a sufficient immunosuppression by TCZ monotherapy. sCD163 has recently been shown to reflect disease activity in a variety of auto-inflammatory diseases, e.g. of Rheumatoid Arthritis [14]. The subnormal values at early time points of our study may be understood as a profound anti-inflammatory effect of combined GC and TCZ, whereas the gradual increase to normal values under TCZ monotherapy suggests reconstitution of homeostasis. A very recent study reports about serum osteopontin as a biomarker of disease activity in GCA and a potential predictor of relapse [15]. Remarkably, our findings show an identical range of initial values and comparable values in remission, however significance was not reached. Due to the lack of relapses, we cannot comment about the value of this biomarker to predict relapse. Collectively, the data of these biomarkers document an ongoing subclinical disease activity with quantitative and qualitative changes over time.

Neutralization of IL-6 blunts the acute phase response. The fact that control of the IL-6 pathway using TCZ is sufficient to induce remission in immunologically complex diseases such as rheumatoid arthritis strongly argues for additional indirect effects, e.g. on the recruitment of pathogenic T helper cells. Indeed, studies have documented that TCZ abrogates generation of TH17 cells in rheumatoid arthritis [16]. In GCA, TH1 and TH17 cells as well as the CD161+CD4+ precursor cells have been shown to be massively increased in the arterial wall, whereas Treg cells are reduced locally and in blood [17]. GC treatment suppresses the TH17 but not the TH1 arm in the blood and the vascular lesions [18]. Very recent data showed that

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TCZ normalizes deranged function of Treg cells [19]. The fact that we could detect neither IL-17 nor any TH1 cytokines in sera is in line with these findings.

Metalloproteinases play an important role in vessel wall inflammation and destruction of elastic fibres [20,21]. IL-6 has been shown to induce production of the tissue inhibitor of metalloproteinases (TIMP) [22,23], and GC have been shown to induce MMP-3 [24]. These findings imply that neutralization of IL-6 as well as treatment with GC may lead to uncontrolled activity of catabolic enzymes and propagation of vessel wall destruction, and - in addition - they may serve as an explanation for the known risk of perforation of diverticulitis under dual immunosuppression in GCA. In this regard, the measured levels of metalloproteinase 3 are of particular interest. As displayed in figure 2, the values show a uniform pattern over time with little inter-individual variation. Furthermore, their values decrease significantly towards study end. Most intriguingly, the MMP-3 values in week 4 are indistinguishable from the values prior to the first infusion of TCZ, although all patients were in full remission at this time point. The values at week 4, therefore, reflect the combined effect of TCZ plus 0.6mg/kg bodyweight of prednisone, whereas at week 52 patients are on TCZ monotherapy. Taken together, these data argue that targeted inhibition of the IL-6 pathway is sufficient to control destructive effector mechanisms. The contradictory results from cell studies and our clinical trial can be reconciled by the hypothesis of indirect effects of TCZ, which eventually overrule the direct effects of IL-6 on TIMP expression.

The correlations between sera concentrations of sTNFR2, ICAM-1, pentraxin-3, CD163, MMP-3 and MRA signals revealed an interesting association between MMP-3 levels and mural signal intensity. Higher levels of pentraxin-3 and lower levels of IL-6 showed a weak correlation with relapse within 6 months after study end. Collectively, however, the data regarding MRA signals and relapse do not suggest that one or a set of analysed biomarkers will emerge as a clinical tool to guide treatment.

They main weakness of this study is the small sample size and the lack of a control population. This is partially counterbalanced by the fact that patients are characterized in great detail and sera were collected in the context of an RCT.

In summary our study documents an extended and persistent subclinical disease activity in GCA which is more pronounced at early stages of treatment and almost disappears towards study end. Inhibition of the IL-6 pathway is not only abolishing signs and symptoms of the systemic inflammation, but prolonged treatment with TCZ appears to reset a range of inflammatory mechanisms. The data are in line with recent findings about effects of TCZ on Th-17 and Treg generation and function.

Declaration of interests

The authors declare no conflicts interests.

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Figure legends

Figure 1. Serum concentrations of TCZ, IL-6 and sIL-6. Serum concentration at each sampling time point with mean ± SD (± SE for sIL-6R) of 17 patients treated with TCZ

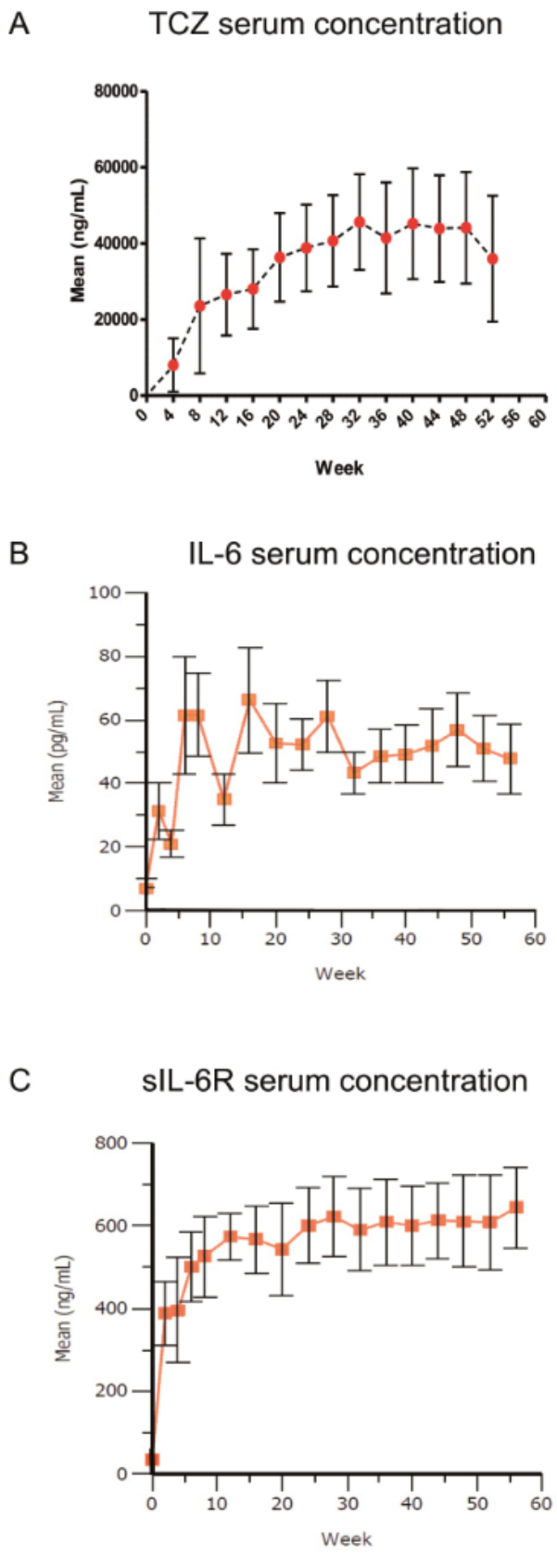

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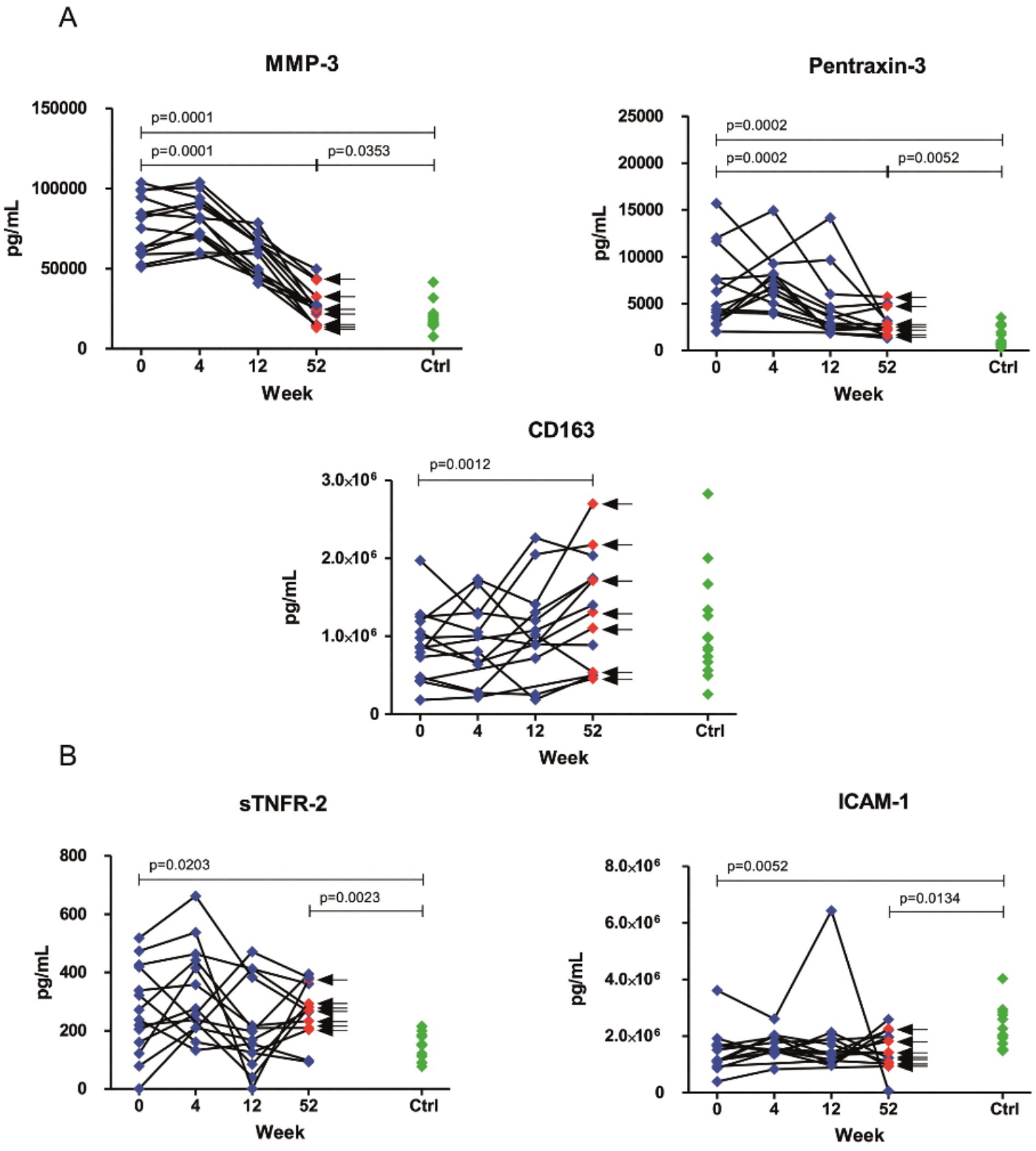
Figure 2. Serum concentration of sTNFR2, ICAM-1, CD163, MMP-3 and pentraxin-3. Serum concentration from 14 patients treated with TCZ over the time in comparison with 14 age- and sex-matched healthy volunteers (Ctrl). Patients with relapse after study end are marked in red and with arrows



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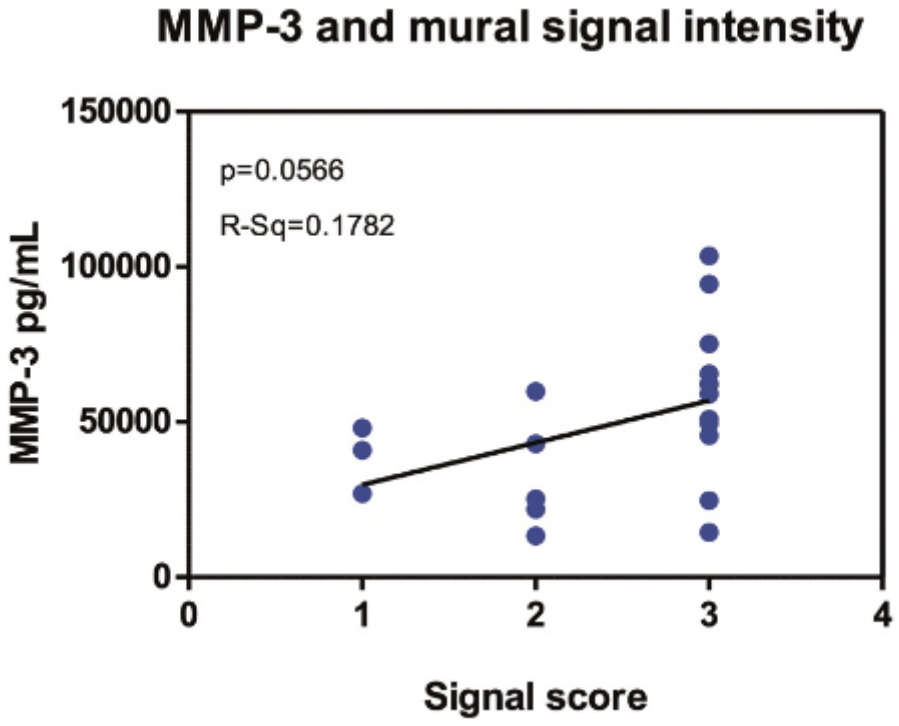
Supplementary figure 1. Correlation between level of MMP-3 and mural signal intensity in MR-angiography



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Tables

Table 1: Spearman's rank correlation between different biomarker and TCZ serum levels

	ICAM-1	sTNFR2	CD163	pentraxin-3	MMP-3	IL-6
Toclizumab serum level	ns	ns	ns	**p=0.0044 r <-0.25**	**p<0.0001 r <-0.5**	**p<0.0001 r >0.5**
IL-6	ns	**p=0.0087 r <-0.25**	**p=0.0208 r >0.25**	**p=0.0033 r <-0.25**	**p<0.0001 r <-0.5**	
MMP-3	ns	ns	ns	**p=0.0005 r >0.25**		
pentraxin-3	ns	ns	**p=0.0403 r <-0.25**			
CD163	ns	ns				
sTNFR2	ns					

a. r= Spearman's rank coefficient; positive correlation if r>0 and negative correlation if r<0
b. ns= correlation not significant

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1 supplementary table

undetectable Biomarkers (N=18)		Biomarkers with non-significant change over time (N=23)		Biomarkers with significiant change over time (5)
IL-2	IL-34	APRIL / TNFSF13	MMP-2	Pentraxin-3
IL-10	IL-32	BAFF / TNFSF13B	ICAM-1	MMP-3
IL-11	IL-29/IFN-λ1	sCD30 / TNFRSF8	hsCRP	sCD163
IL-12(p40)	IL-28A/ IFN-λ2	Chitinase-3-like1/YKL-40	Osteopontin	IL-6
IL-12(p70)	IL-27(p28)	gp130 / sIL-6Rβ	sTNFR1	sIL-6R
IL17A	IFN-γ	IFN-α2	sTNFR2	
IL-20	MMP-1	IFN-β	TSLP	
IL-22	Osteocalcin	sCD25/IL-R2	TWEAK / TNFSF12	
IL-26	VCAM-1	IL-8	Leptin	
		IL-19	Resistin	
		IL-35	Adiponectin	
		LIGHT / TNFSF14		

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